

Localization of an arginine-specific mono-ADP-ribosyltransferase in skeletal muscle sarcolemma and transverse tubules

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Received 1 February 1994; revised version received 18 February 1994

Abstract

The precise localization of a membrane-bound, arginine-specific mono-ADP-ribosyltransferase (mADP-RT) was assessed in rabbit skeletal muscle by studying membrane fractions isolated by successive sucrose density gradient centrifugations. mADP-RT activity was 10-fold enriched in sarcolemmal and T-tubular membranes. The catalytic activity, determined in preparations with mainly right-side-out vesicles, was found to be on the cytoplasmic face. As revealed by SDS-PAGE and autoradiography endogenous mADP-RT activity labeled several proteins in the range between 15 kDa and 250 kDa. T-tubules contained the highest number of [³²P]ADP-ribose-labeled proteins.

Key words: Mono-ADP-ribosyltransferase; Rabbit skeletal muscle; Sarcolemma; T-tubule

1. Introduction

ADP-ribosylation by poly-ADP-ribosyltransferases (EC 2.4.2.30) or mono-ADP-ribosyltransferases (mADP-RT, EC 2.4.2.31) represents a post-translational modification. Since the initial report of Moss and Vaughan [1], several endogenous mADP-RTs have been identified in animal tissues, including skeletal and cardiac muscle [2], and are classified according to various acceptor amino acid residues [3]. The reverse reaction of arginine-specific ADP-ribosylation is catalyzed by an ADP-ribosylarginine-hydrolase (EC 3.2.2.14) [4].

High mADP-RT activities have been detected in crude sarcoplasmic reticulum (SR) and sarcolemmal preparations from pig and rabbit-skeletal muscles [2,5,6]. Hara et al. [7] reported endogenous ADP-ribosylation of SR Ca²⁺-ATPase, leading to an inhibition of its ATP-hydrolyzing activity. A possible regulatory role of mADP-RT was also suggested by mono-ADP-ribosylation of several other microsomal proteins [8–10]. In view of the as yet unidentified function of mADP-RT in cross-striated muscle, the exact location of this enzyme needs to be elucidated. Crude SR preparations are composed

of vesicles from several membrane compartments with distinct protein profiles and functions, such as sarcolemma, T-tubules, terminal cisternae, and longitudinal SR. The present study was undertaken to assign mADP-RT activity to defined membrane compartments. For this purpose, crude SR was fractionated by sucrose density centrifugation and mADP-RT activity determined in fractions identified by marker enzymes and specific ligands. Our data show that mADP-RT is predominantly present in vesicles derived from the T-tubules and sarcolemma and that its catalytic activity is oriented towards the cytoplasm.

2. Materials and methods

2.1. Materials

Biochemicals and chemicals were from Sigma (alamethicin, agmatine, cholera toxin subunit A, isoniazin, novobiocin, thymidine, all detergents). Boehringer Mannheim (NAD⁺, NADH, guanosine 5'-[γ-thio]triphosphate) and Bio-Rad (Dowex AG1-X2 resin, mesh size 100–200). [¹⁴C]Nicotinamide, [¹⁴C]NAD⁺ and [³²P]NAD⁺ were from Amersham.

2.2. Membrane fractionation

Crude microsomes isolated from rabbit back muscles were fractionated by centrifugation on linear sucrose gradients (0.74–1.4 M) [11], yielding fractions B1, B2, B3 (from heaviest to lightest). Fraction B1 was collected and applied to an ion-free linear sucrose gradient (0.74–1.4 M), containing 0.05 mM PMSF. The second centrifugation yielded three fractions, named (from heaviest to lightest) B1.1, B1.2 and B1.3. Sarcolemmal membranes were isolated according to Ohlndieck et al. [12].

2.3. Enzyme activities

Mg²⁺-ATPase (EC 3.6.1.3), Ca²⁺-ATPase (EC 3.6.1.38), ouabain-sensitive (Na⁺,K⁺)-ATPase (EC 3.6.1.37) and lactate dehydrogenase

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Abbreviations: C₁₂E₉, polyoxyethylene 9 lauryl ether; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; GTP[S], guanosine 5'-[γ-thio]triphosphate; lysoPtdCho, lysophosphatidylcholine; mADP-RT, arginine-specific mono-ADP-ribosyltransferase; PMSF, phenylmethylsulfonyl fluoride.

Table 1

Biochemical properties of membrane fractions obtained by sucrose density gradient centrifugation

Fractions	Ca ²⁺ -ATPase ($\mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$)	Mg ²⁺ -ATPase ($\mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$)	(Na ⁺ ,K ⁺)-ATPase ($\text{nmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$)	Ryanodine binding ($\text{pmol} \cdot \text{mg}^{-1}$)	PN200-110 binding ($\text{pmol} \cdot \text{mg}^{-1}$)	Saxitoxin binding ($\text{pmol} \cdot \text{mg}^{-1}$)
B1	18.9	0.7	26	9.7	3.2	0.2
B2	21.3	1.0	140	5.4	3.1	0.3
B3	3.2	3.4	275	0.6	18.4	1.2
B1.1	14.4	0.5	14	7.0	2.6	0.5
B1.2	24.5	0.5	10	0.8	2.6	0.3
B1.3	3.0	1.9	129	0.2	22.2	0.6
SL	4.5	2.8	336	0.3	3.1	2.3

The identity of B1, B2, B3, B1.1, B1.2, B1.3 is as described in section 3. SL is an abbreviation for sarcolemmal vesicles, isolated according to [12]. Values are representative of 7 independent experiments.

(EC 1.1.1.27) activities were measured photometrically [13]. Latent Mg²⁺-ATPase and (Na⁺,K⁺)-ATPase activities were unmasked by alamethicin (1 mg/mg protein) and saponin (70 mg/mg protein). Acetylcholinesterase (EC 3.1.1.7) activity was measured according to [14]. 0.1% (v/v) Triton X-100 unmasked its latent activity. Both, mADP-RT and NAD⁺ glycohydrolase (EC 3.2.2.5) activities were determined using the NAD⁺ glycohydrolase assay [15]. The reaction mixture contained 140 μl of 50 mM potassium phosphate (pH 7.0), 2 mM [*carbonyl*-¹⁴C]NAD⁺ (60 cpm/nmol) and the indicated amount of membranes or purified proteins, with or without 20 mM agmatine as an acceptor for ADP-ribose. Detergents (C₁₂E₉, 0.3 mg/ml) were added for membrane solubilization. After 60 min incubation at 30°C, 100 μl aliquots were applied to Dowex AG 1-X2 columns and washed with 5 ml of 20 mM Tris-HCl (pH 7.3). The eluted [*carbonyl*-¹⁴C]nicotinamide was collected into vials containing 10 ml Ultima Gold XR (Canberra-Packard). The radioactivity was measured in a liquid scintillation counter. NAD⁺ glycohydrolase activity determined in the absence of agmatine was subtracted from the combined NAD⁺ glycohydrolase and mADP-RT activities measured in the presence of agmatine. The difference yielded the activity of the mADP-RT. The recovery rate of [*carbonyl*-¹⁴C]nicotinamide was evaluated by using genuine [*carbonyl*-¹⁴C]nicotinamide and was taken into account for calculating specific activities. Recovery reached almost 98%. The validity of this assay was assured by using cholera toxin subunit A1 as an authentic mADP-RT [15]. In some experiments, a membrane filter assay [16] was used for measuring mADP-RT activity.

2.4. Binding studies

Binding of [³H]ryanodine, [³H]PN200-110, and [³H]saxitoxin to membrane fractions was determined according to [17–19].

2.5. Light scattering

Light scattering (excitation at 400 nm, emission at 407 nm) was measured in order to study the effect of detergents on membrane solubilization [20]. CHAPS, lysophosphatidylcholine (lysoPtdCho), *n*-octylglucoside, polyoxyethylene 9 lauryl ether (C₁₂E₉) sodium deoxycholate and saponin were chosen as detergents.

2.6. ADP-ribosylation of membranes

Endogenous mono-ADP ribosylation was conducted in a medium containing 50 mM Tris-HCl (pH 7.9), 10 mM MgCl₂, 1 mM EDTA, 1 mM ATP, 0.5 mM GTP[S], 10 mM thymidine, 20 mM isoniacin, 0.1% (v/v) Triton X-100, 0.2 mM PMSF, 5 μM [*adenylate*-³²P]NAD⁺, (0.5–4.5 μCi /tube) and 40 μg membrane protein. Novobiocin, an inhibitor of arginine-specific mADP-RT [21], was included in some experiments at 4 mM concentration. After 30 min at 37°C, the reaction was stopped by adding ice-cold trichloroacetic acid to a final concentration of 20% (v/v). The precipitated protein was washed twice with diethylether, solubilized and processed for electrophoretic analysis [22]. Gels loaded with radioactive samples were analysed by autoradiography or by phosphor-imaging.

3. Results

3.1. mADP-RT activities of membrane fractions

Membrane fractions were characterized by SDS-PAGE (data not shown), marker enzyme activities, and binding of specific ligands (Table 1). Ca²⁺-ATPase was taken as a marker for longitudinal SR, Mg²⁺-ATPase and (Na⁺,K⁺)-ATPase for both sarcolemma and T-tubules. Ryanodine binding indicated the ryanodine receptor in the terminal cisternae as components of the triads. The binding of PN200-110 to the dihydropyridine receptor was specific of T-Tubules, especially of junctional T-tubules. Finally, saxitoxin binding to the sodium channel was taken as a sarcolemmal marker. Thus, fractions B1, B2, B3 were enriched in triads, longitudinal SR and free T-tubules, respectively. The fractions obtained after centrifugation of the heaviest fraction (B1), consisted predominantly of terminal cisternae (B1.1), longitudinal SR (B1.2) and junctional T-tubules (B1.3).

As membrane fragments vesiculate in aqueous solutions, specific mADP-RT activities were determined in the presence of detergents (lysoPtdCho, *n*-octylglucoside, C₁₂E₉), characterized as suitable for membrane sol-

Table 2

Specific activity of mADP-RT in subfractions from density gradient centrifugations of rabbit skeletal muscle microsomes

Fractions	mADP-RT activity ($\text{nmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$)
B1	2.78 $\pm$ 1.3
B2	3.36 $\pm$ 1.7
B3	22.48 $\pm$ 5.0
B1.1	1.33 $\pm$ 0.9
B1.2	2.26 $\pm$ 1.0
B1.3	20.7 $\pm$ 6.2
SL	18.68 $\pm$ 0.9

Enzyme activity was determined in the presence of C₁₂E₉ or lysoPtdCho (detergent to protein ratio of 10 to 1) using the NAD⁺ glycohydrolase assay. Fractions are named as indicated in Fig. 1. The values are means $\pm$ S.D. from 7 independent preparations.

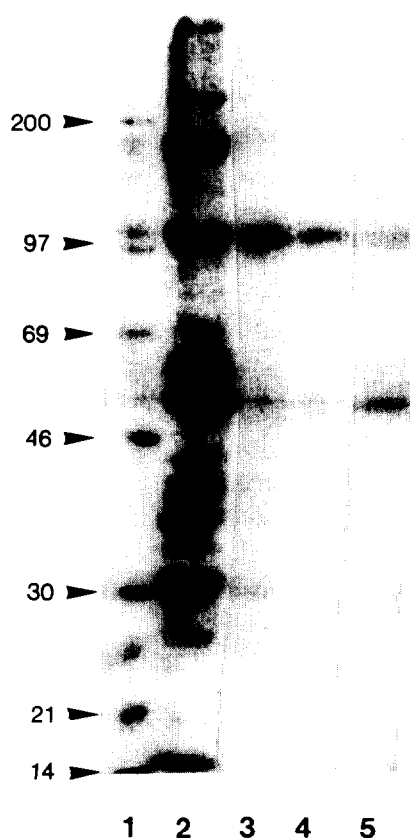


Fig. 1. Autoradiography (phosphoimaging) of SDS-PAGE performed on various membrane fractions after mono-ADP-ribosylation in the presence of [32 P]NAD $^{+}$. Lane 1, 14 C-methylated protein marker; lanes 2 and 5, free T-tubules (fractions B3); lane 3, terminal cisternae (fraction B1.1); lane 4, longitudinal sarcoplasmic reticulum (fraction B1.2). The incubation shown in lane 5 contained 4 mM novobiocin, a specific inhibitor of arginine-specific mADP-RT [21].

ubilization by changes in light-scattering [20]. Saponin had no effect on light scattering. At concentrations below the critical micellar concentration, all detergents induced identical increases in mADP-RT activity, most probably resulting from membrane perforation. However, higher concentrations of Chaps and sodium deoxycholate reduced mADP-RT activity. Sarcolemmal and T-tubular fractions contained 10-fold higher mADP-RT activities than the other fractions (Table 2). Higher mADP-RT activities in T-tubules and sarcolemma than in the other membrane fractions were also detected by autoradiography of SDS-PAGE after endogenous mono-ADP-ribosylation (Fig. 1) and by the membrane filter assay using histone as an acceptor (data not shown).

3.2. Orientation of mADP-RT in T-tubular and sarcolemmal vesicles

mADP-RT activity in membrane vesicles increased significantly after treatment with an appropriate detergent. Taking into account that the vesicles exhibited a defined sidedness, this finding suggested a specific orientation of the catalytic site. The integrity and sidedness of the membranes was assessed by measuring patent and total activities of marker enzymes in the absence and presence of unmasking agents (Table 3). Mg $^{2+}$ -ATPase activity, recently shown to be oriented with its active site on the outside of the T-tubule [13,23], increased slightly in the presence of alamethicin. Small increases in the presence of unmasking agents were also observed for other ecto-enzymes, e.g. acetylcholinesterase [24] and NAD $^{+}$ glycohydrolase [25] (Table 3).

The percentage of leaky vesicles was estimated from patent and total lactate dehydrogenase and (Na $^{+}$,K $^{+}$)-ATPase activities [13,24]. Approximately 20% of the T-

Table 3

Integrity and sidedness of sarcolemma, free and junctional T-tubules as evaluated by measurement of marker enzyme activities with and without unmasking agents

Marker enzymes	Patent activity	Total activity	Vesicle subpopulation without unmasking	
			Class	% of total population
Free and junctional T-tubules				
Mg ²⁺ -ATPase	1730	1930	L + RSO	89.6
Acetylcholinesterase	6.6	8.0	L + RSO	82.5
NAD ⁺ glycohydrolase	62.9	73.7	L + RSO	85.4
Lactate dehydrogenase	1160	5640	L	20.6
(Na ⁺ ,K ⁺)-ATPase	46.6	262	L	17.8
Sarcolemma				
Mg ²⁺ -ATPase	2860	2840	L + RSO	100
Acetylcholinesterase	7.6	7.6	L + RSO	100
NAD ⁺ glycohydrolase	87.7	86.9	L + RSO	100
Lactate dehydrogenase	607	2825	L	21.5
(Na ⁺ ,K ⁺)-ATPase	94.2	384	L	24.5

Values are representative of 7 independent experiments. Enzyme activities are given as nmol $\cdot$ mg $^{-1}$ $\cdot$ min $^{-1}$. Abbreviations: L, leaky vesicles; RSO, right-side out vesicles. Alamethicin (1 mg/mg protein) unmasked Mg $^{2+}$ -ATPase and (Na $^{+}$,K $^{+}$)-ATPase, Triton X-100 (0.1%) acetylcholinesterase, C $_{12}$ E $_9$ (0.3 mg/ml) NAD $^{+}$ glycohydrolase, and saponin (0.2 mg/ml) lactate dehydrogenase.

tubular vesicles were leaky (Table 3). Total enzyme activity determined in the presence of unmasking agent was taken as the sum of inside out, right-side out and leaky vesicles. According to Table 3, 65–70% of the vesicles were sealed and right-side out, whereas 15–20% were leaky, leaving 10–15% sealed and inside-out. This proportion was the same in free and junctional T-tubules. Sarcolemmal membranes displayed 75–80% right-side out and 20–25% leaky vesicles. These percentages could be reproduced by using detergents that did not impair the catalytic activities of the enzymes under study, e.g. C₁₂E₉. Approximately 25% and 20% of the T-tubular and sarcolemmal mADP-RT activity were patent (Table 4). These values agreed with the percentage of both leaky and inside out vesicles, suggesting an orientation of the catalytic site on the cytoplasmic face of T-tubular and sarcolemmal membranes.

3.3. Mono-ADP-ribosylation of proteins in various membrane fractions

As compared to T-tubular membranes, proteins from terminal cisternae and longitudinal SR showed much smaller incorporation of [³²P]ADP-ribose (Fig. 1) which agreed with the lower specific mADP-RT activities of these two fractions (Table 2). In terminal cisternae, weak bands were detected at 30 kDa, 55 kDa and 102 kDa (Fig. 1). Longitudinal SR displayed a similar profile, except for the absence of the 30 kDa band. T-tubules contained the 30 kDa, 55 kDa and 102 kDa bands and labeled proteins at 15, 26, 37, 39, 43, 60, 69, 160 and 250 kDa. The 55 kDa, 60 kDa, 102 kDa and 160 kDa bands exhibited the highest incorporation. Radioactive labeling depended on arginine-specific mono-ADP-ribosylation because novobiocin, a specific inhibitor of the enzyme [21], almost completely abolished the incorporation of the label (Fig. 1, lane 5).

4. Discussion

Our studies on mADP-RT in skeletal muscle point to

a function of this enzyme in the sarcolemma and the T-tubules. An enrichment of mADP-RT by an order of magnitude in these membranes corresponds to similar increases of marker enzyme activities and ion channel ligands specific to sarcolemma and T-tubules. This localization is confirmed by severalfold higher incorporation of ADP-ribose from [³²P]NAD⁺ into proteins of the T-tubule membrane as compared to proteins of longitudinal SR and terminal cisternae. The specificity of arginine-specific mono-ADP-ribosylation is documented by the inhibitory effect of novobiocin, a specific inhibitor of mADP-RT [21].

The data on the sarcolemmal distribution are in accordance with reports on mono-ADP ribosylation in cardiac and skeletal sarcolemma [5,26,27]. Here we provide evidence for an additional location of mADP-RT, i.e. in the T-tubules. Its specific activity in junctional T-tubules, derived from sarcolemma-free triads, exceeds that of the sarcolemma. Our T-tubule and sarcolemma preparations consist mainly of sealed vesicles with right-side out orientation, which agrees with previously published data [13,23]. The patency of mADP-RT in T-tubule preparations represents 25% of its total activity. This value corresponds to the sum of sealed inside-out and leaky vesicles. The possibility that the enzyme is trapped by vesiculation is excluded by the fact that mADP-RT is membrane-anchored [6,27,28]. Treatment with detergent unmasks approximately 75% of its total activity, suggesting that the catalytic site of the enzyme is on the cytoplasmic surface of the membrane. The possibility that mADP-RT is directly activated by detergents can be excluded since membrane perforation by saponin and low detergent concentration lead to identical activations. Also, the detergents used have no stimulatory effect on the purified enzyme (data not shown).

Our findings are not in favour of an extracellular orientation of the sarcolemmal mADP-RT as recently suggested for a sarcolemmal mADP-RT in two mouse cell lines [28]. With the exception of the presumed integrin [28], all putative target proteins identified to date are intracellular proteins, e.g. G-proteins [29]. cAMP-independent protein kinase [26], proteins assigned to the SR [7,8,10] or the T-tubule [30]. Our results disclose additional target proteins of endogenous mono-ADP-ribosylation. These are more numerous in the T-tubules than in longitudinal SR and terminal cisternae (Fig. 1). Finally, the cytoplasmic orientation of the membrane-bound mADP-RT (this study) together with an intracellular ADP-ribosylarginine hydrolase [31] point to the existence of a mono-ADP-ribosylation cycle [32] in skeletal muscle.

Table 4

Patent and total mADP-RT activities in T-tubules and sarcolemma as determined by the NAD⁺ glycohydrolase assay in the absence and presence of lysoPtdCho or C₁₂E₉ at a detergent to protein ratio of 10 to 1

Fractions	Activity (nmol · mg ⁻¹ · min ⁻¹)		% latency
	Patent	Total	
Free T-tubules	7.5	30.6	24.5
Junctional T-tubules	6.2	26.1	23.8
Sarcolemma	3.7	19.1	19.4

Values are representative for 7 independent experiments.

Acknowledgements: This study was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 156 and DFG Grant Pe 62/19-2. We are grateful to Dr. B. Brüne for helpful suggestions. S.M. was a recipient of a fellowship from the Alexander von Humboldt Foundation.

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